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Radial distribution of heartwood phenols and the cytological changes of ray parenchyma cells associated with heartwood formation in Japanese red pine (*Pinus densiflora* Sieb. et Zucc.)

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アカマツの心材形成における心材フェノール成分の特徴と細胞学的変化

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Résumé

To clarify the characteristics of heartwood formation in Japanese red pine (*Pinus densiflora*) radial distribution of heartwood phenols and cytological structure were investigated. The results were: (1) The boundary between sapwood and heartwood was distinct although the intermediate wood (or white zone) was not recognized. (2) Methanol extractives were small amount in sapwood and increased in the transition zone from sapwood to heartwood. (3) From the analysis of methanol extractives by thin layer chromatography it was revealed that pinosylvin and its monomethyl ether were the two main heartwood phenols. In sapwood they were in trace amount but they increased abruptly in the transition zone. (4) The percentage of living ray parenchyma cells was almost 100% in the sapwood and in the transition zone from sapwood to heartwood (within one annual ring) it decreased to 0%. (5) Main reserve substance was lipids. In sapwood they showed neutral lipidic nature. In the transition zone they abruptly changed quantitatively and qualitatively. (6) Moisture content was high in sapwood and it decreased in heartwood. Comparing June and October it was a distinctive feature that higher moisture content in the inner sapwood in June decreased in October. (7) From the characteristics mentioned above it was revealed that all of the investigated features showed abrupt changes in the very narrow region between sapwood and heartwood. From the comparison of the characteristics between June and October it was considered that heartwood formation progressed after summer. From the comparison of the features of heartwood formation between Japanese red pine and Japanese cedar, moreover, it was concluded that the investigated characteristics of Japanese red pine basically resembled to those of Japanese cedar.

要　　旨

アカマツ（*Pinus densiflora*）の心材形成の特徴について，心材フェノール成分の放射方向における分布とエイジングに伴う細胞学的変化の観察から検討した。得られた結果は以下の通りである。(1)供試材の辺・心材境界は明瞭であり，移行材（または白線帯）は含まれていなかった。(2)メタノール抽出物は辺材では少なく，辺・心材境界で急激に増加した。(3)メタノール抽出物の薄層クロマトグラフィーによる分析から，ピノシルビン及びそのモノメチルエーテルが心材での主成分であることがわかった。また，これらは辺・心材境界で急激に形成された。(4)放射柔細胞

の生存率は辺材部を通して辺・心材境界までほぼ100%であった。辺・心材境界の狭い範囲（1年以内）で，0%に減少した。(5)貯蔵物質は脂質が中心であった。これらは辺材部で中性脂質の性質を示したが，辺・心材境界で急激に他の成分へと変化したと推定された。(6)含水率は辺材部で高く，心材部で減少した。また，6月と10月を比較すると，辺材内層部で含水率が6月に比べ10月に減少することが特徴的であった。以上の結果から，(7)アカマツの心材形成に関わる変化は，辺・心材境界の非常に限られた領域で急激に起こっている事が判明した。しかし，6月と10月の比較から夏以降に心材化が進むこと，また辺・心材境界で起こっている特徴は，スギ等における心材形成の特徴と類似していること，が推定された。

1. Introduction

For the elucidation of the mechanism of heartwood formation it is important to investigate both cytological characteristics of ray parenchyma cells and heartwood substances and to discuss the relationship between them. Nobuchi and others[1] reported the characteristics of heartwood formation in Japanese cedar (*Cryptomeria japonica* D. Don) from this view point.

The type of heartwood is generally species-dependent. From the report[2] concerning the types of heartwood which were investigated from the radial changes of the proportion of living ray parenchyma cells, Japanese red pine showed different pattern from that of Japanese cedar. One of the possible reasons is that the latter has a clear intermediate wood but the former generally does not have it. In addition to the intermediate wood the main reserve substance of the former is lipids different from the latter in which starch is the main reserve substance[3].

In this report, therefore, the characteristics of heartwood formation in Japanese red pine has been investigated. The discussion of the features of heartwood formation in Japanese red pine has been carried out in comparison with Japanese cedar.

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2. Materials and methods

2.1 *Materials*

Two Japanese red pine (*Pinus densiflora* Sieb. et Zucc.) trees which grew in the natural forest of Wakayama Experimental Forest of Kyoto University were selected for the sample trees. The conditions of sample trees and the dates of felling are shown in Table 1. In one sample tree (Tree A), sample blocks were taken in two seasons to investigate seasonal features of heart-

Table 1 Description of sample trees*

Tree name	Tree A	Tree B
Age (year)	65	66
Height (m)	18	17
D. B. H. (cm)	46	49
Date of taking sample	June 6, 1986 (by an increment borer**) Oct. 9, 1986 (Felling)	June 6, 1986 (Felling)

* Wood blocks were taken from the breast height
** Wood blocks containing sapwood and outer heartwood were collected

wood formation, that is in June which is thought to be the pre-stage of heartwood formation and in October which is to be in the process of heartwood formation.

2.2 *Methods*

Sample blocks taken from the living tree were immediately treated as follows:

2.2.1 *Analysis of heartwood phenols*

The fresh sample block containing cambium to pith was divided into small blocks and each small block was soaked in methanol. The method of phenol analysis was based on Mibayashi and others[4]. The developer in thin layer chromatography (TLC) was benzene and acetone (4:1). Two major heartwood phenols of Japanese red pine, pinosylvin and pinosylvin monomethyl ether, were analyzed using a chromatoscanner (Shimadzu, CS-920). The maximum ultraviolet (UV) absorbance was 301nm for both phenols. The data were shown as the average of three measurements.

2.2.2 *Moisture content*

The radial change of moisture content was investigated. The division of sample blocks from cambium to pith was the same as for the analysis of heartwood phenols.

2.2.3 *Light microscopy*

Wood blocks containing cambium to pith were fixed immediately with 3% glutaraldehyde (phosphate buffer, pH 6.98). Radial sections 20μm in thickness were cut covering from cambium to pith and were stained as follows: Safranin and light green for nuclei, Nile blue or Sudan IV for lipid droplets, and I_2-KI for starch grains. The radial changes of the percentage of living ray parenchyma cells and the changes in reserve substances — lipid droplets, starch grains — in the transition zone from sapwood to heartwood were mainly investigated.

3. Results and discussion

3.1 *Characteristics of xylem color between sapwood and heartwood*

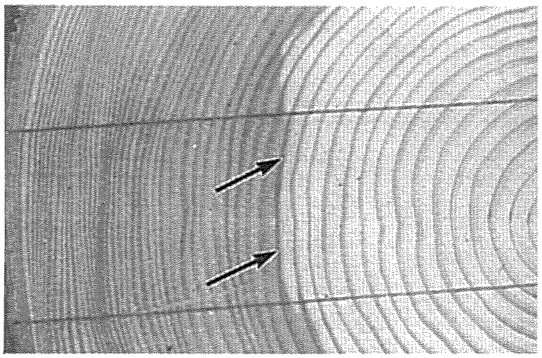


Fig. 1 A transverse section of Japanese red pine showing the boundary between sapwood and heartwood (arrows). No intermediate wood is observed (Tree B, June).

The heartwood color of both sample trees showed pale brown and an intermediate wood (or white zone) was not recognized between sapwood and heartwood (Fig. 1). Figure 1 was taken just after felling. The sapwood shows darker color in a black and white print because of the high moisture content of sapwood. In this study, therefore, the xylem from cambium to pith was divided into sapwood and heartwood based on the wood color.

Harris[5] reported the existence of dry wood zone which corresponded to white zone between sapwood and heartwood in *Pinus radiata.* It is possible that there are two cases having and not having intermediate wood in the genus.

3.2 *Radial distributions of methanol extractives and heartwood phenols*

A radial distribution pattern of methanol extractives in tree A (October) is shown in Fig. 2. The quantity of methanol extractives was small in sapwood ranging between 1-2%, increased in the transition zone from sapwood to heartwood, and was large in heartwood region. It was also characteristics that they increased towards pith in heartwood region. The methanol extractives are considered to include phenols, methanol soluble lipids and others. The proportion of lipids and oleoresin in the methanol extractives is thought to be high in sapwood because *Pinus densiflora* belongs to "fat tree" and has resin canals. Similar radial distribution patterns of methanol extractives were obtained in Tree A (June) and Tree B (data are not shown).

On TLC plate two main spots showed strong absorbance in UV range in the measurement of methanol extractives by a chromatoscanner. After comparing them with standard phenols, it was determined that they were pinosylvin (Rf value=0.44) and pinosylvin monomethyl ether (Rf value=0.74). The determination of the absolute values of two phenols was based on the calibration curves taken from the measurement of standard phenols. Calibration curves are shown in Fig. 3. In Fig. 3 calibration curves showed almost straight lines which cross the origin. Towards high concentration, however, the concentration of phenols is not always proportional to area value, especially in pinosylvin

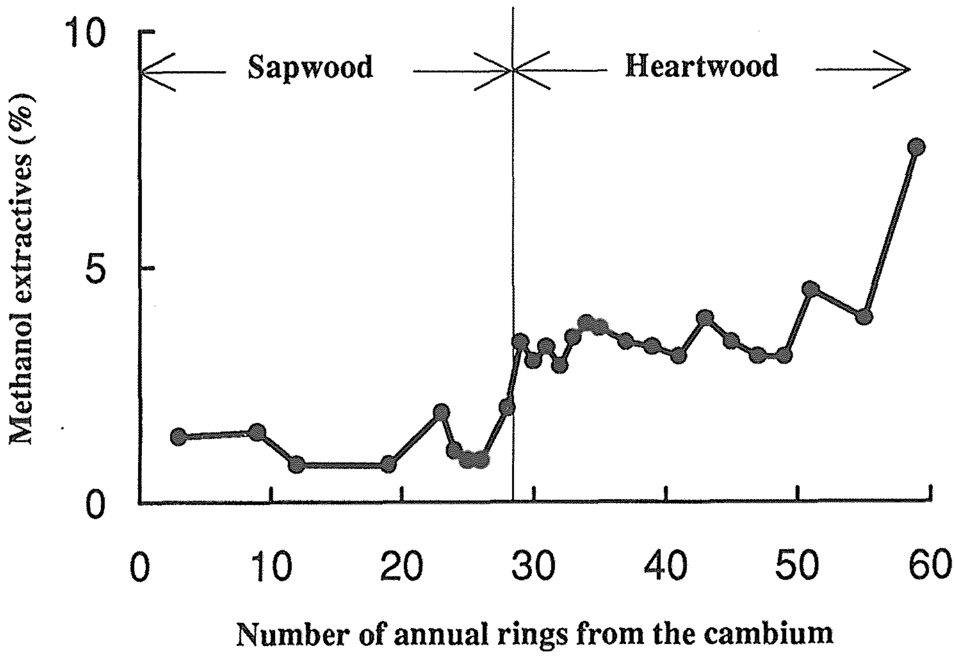
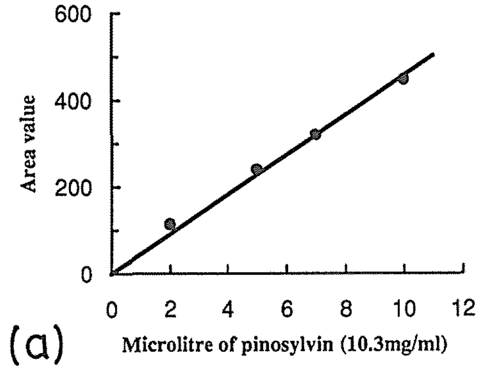


Fig. 2 Radial distribution pattern of methanol extractives (Tree A, October).



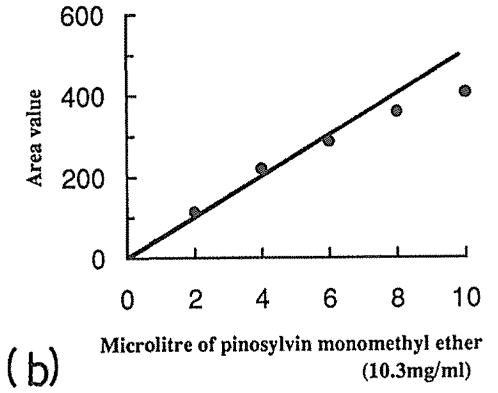


Fig. 3 Calibration curves of pinosylvin (a) and its monomethyl ether (b).

monomethyl ether. It is possible that underestimation will occur towards higher concentration. In the real measurement in TLC, we controlled the concentration of methanol extractives to be put in the range of a straight line of calibration curves.

Radial distribution patterns of two heartwood phenols in Tree A (October) and Tree B (June) are shown in Figs. 4 (a) and 4 (b), respectively. Both pinosylvin and its monomethyl ether showed almost trace amount in sapwood and they increased abruptly in the boundary between sapwood and heartwood. They showed high levels in heartwood although the levels showed fluctuation. The proportion of heartwood phenols was between 0.5-2.0% of the dry weight of wood. Hata[6] reported that the proportion of the total of these two crystallized phenols was 0.36% of dry weight of wood. He also reported that 0.36% of phenol showed only pure crystals and extractives contained more phenols which were not separated as crystals. The data of present experiment, therefore, are considered to be reasonable.

In the studies of the relationship between wood color and extractives in Japanese cypress, Hayashi and others[7] reported that pinky and pale colors of wood coincided with more or less the quantity of hinokiresinol. This result indicates that the rhythm of increase and decrease of extractives occurs in the heartwood region, although the reason is not clear. It is thought in the present trees that the fluctuation of the amount of heartwood phenols occurred for some reasons.

Comparing Tree A and B in Fig. 4, the ratio of two heartwood phenols was different between trees. That is, in Tree B pinosylvin monomethyl ether was much more than pinosylvin but in tree A there was not so much difference. This result reveals that the variability of the ratio of heartwood phenols occurred even if the same kinds of phenols were included in Japanese red pine.

Regarding the seasonal features of heartwood formation it is reported that heartwood formation started in summer season and had its maximum in autumn[8)~10)]. June and October are, therefore, thought to be the pre-stage of heartwood formation and the season of heartwood formation, respectively. To consider the seasonal characteristics of heart-

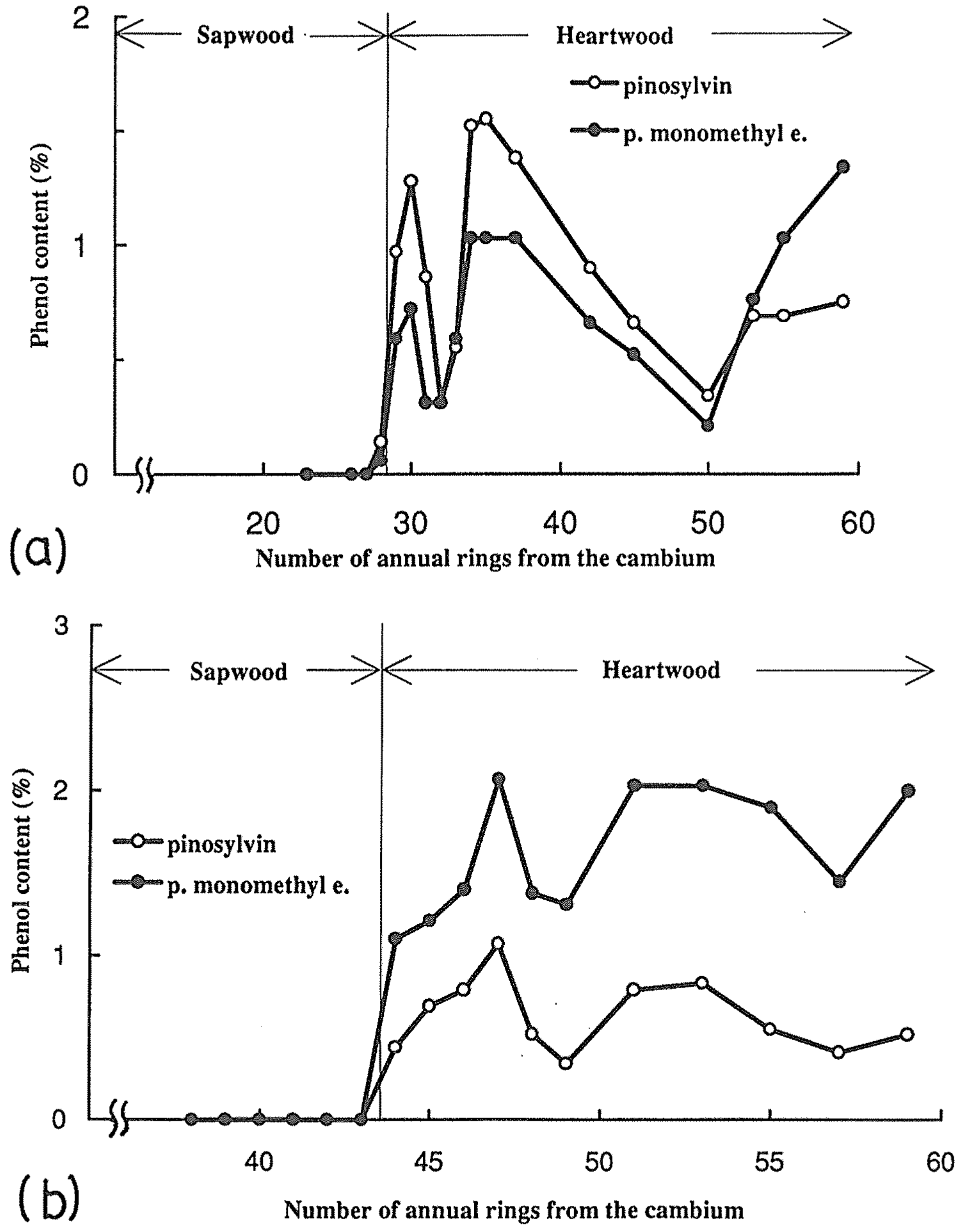


Fig. 4 Radial distribution patterns of pinosylvin and its monomethyl ether.
(a) Tree A, October, (b) Tree B, June.

wood formation from the biosynthesis of heartwood phenols, the amount of each phenol between June and October was compared in tree A. The assumption that the boundary between sapwood and heartwood moved one annual ring ahead towards cambial side during four months from June to October was adopted. That is, the innermost annual ring of sapwood in June was thought to be the outermost annual ring of heartwood in October.

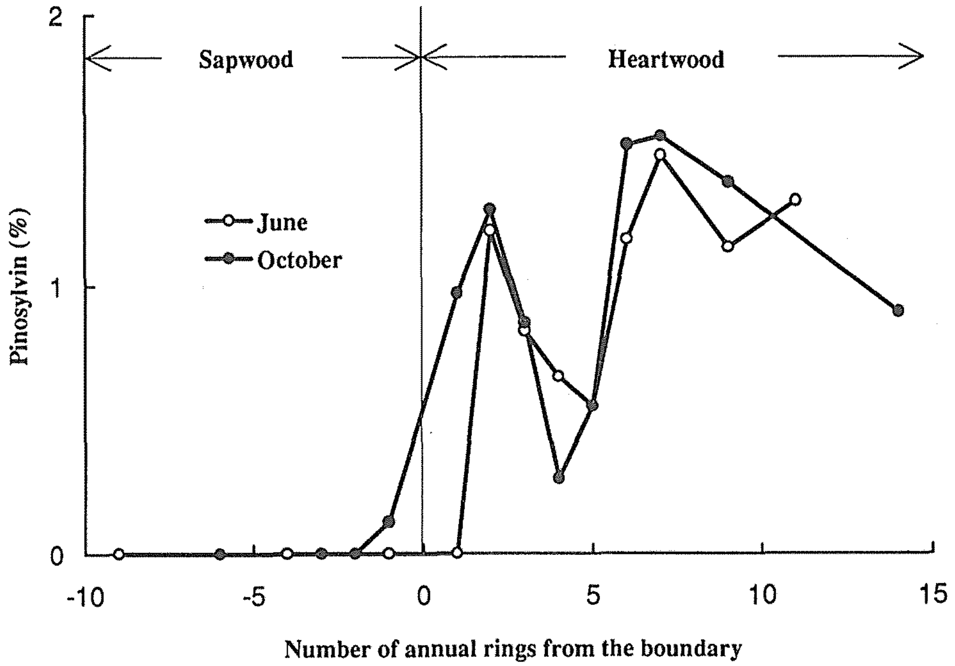


Fig. 5 Copmarison of the radial distribution patterns of pinosylvin between June and October in Tree A.

Figure 5 shows the comparison of heartwood phenol between June and October (only the data of pinosylvin are shown). In Fig. 5, therefore, the horizontal axis standardized based on the boundary between sapwood and heartwood of October.

Comparing the positions of taking sample blocks between June and October, circumferential positions were different although both were taken at the breast height from the ground level. The radial distribution patterns of each phenols, however, showed good coincidence between them. In the outermost annual ring of heartwood in Fig. 5 two phenols showed conspicuous increase from June to October. This indicated that the biosynthesis of heartwood phenols progressed during these months. The season of heartwood formation is thought to be in accordance with the results of past reports[8)~10)] in which the heartwood formation started from summer to autumn.

Only trace amounts of two heartwood phenols were detected in sapwood. Detailed observation revealed that pinosylvin monomethyl ether was included more than pinosylvin. This tendency is thought to be reasonable from the consideration that Rudloff and Jorgensen[11)] presumed that pinosylvin monomethyl ether was the precursor of pinosylvin.

3.3 *Characteristics of cytological structure of ray parenchyma cells associated with heartwood formation*

3.3.1 *Radial changes of the proportion of living ray parenchyma cells*

The proportion of living ray parenchyma cells in each annual ring was measured from cambium to pith based on Ziegler[12)] and Nobuchi and others[2)]. The results in Tree A are

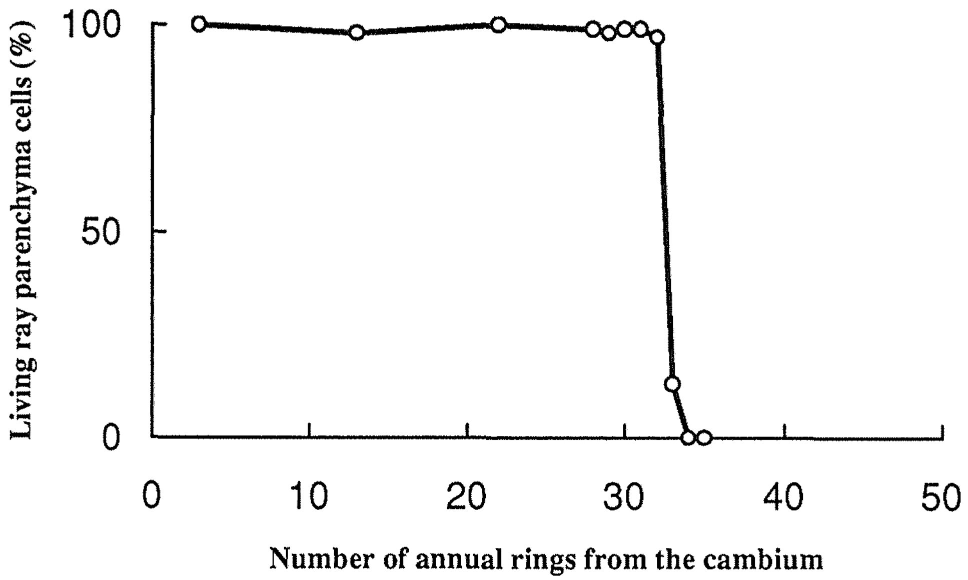


Fig. 6 Radial distribution pattern of the percentage of living ray parenchyma cells (Tree A, October).

shown in Fig. 6. The proportions of living ray parenchyma cells showed almost 100% from outer to inner sapwood and they decreased to 0% within one annual ring in the transition zone from sapwood to heartwood.

From the more detailed observation of radial distribution patterns near the boundary in Tree A (June and October), the proportion of living cells decreased more rapidly in June, and rather gradually in October. The difference between them is considered to be because of the seasonal difference of heartwood formation. That is, in October which is thought to be the mid season of heartwood formation, the proportion of dead cells increased.

The radial width where the proportion of living ray parenchyma cells decreased from 100 to 0%, however, was one annual ring at the most. This narrow width was considered to be characteristics of Japanese red pine differing from, for example, Japanese cedar. In this experiment living ray parenchyma cells existed in the outermost annual ring of heartwood. This tendency is in accordance with the results in Japanese cedar[13].

3.3.2 *Changes of reserve substances associated with heartwood formation*

Main reserve substance in Japanese red pine was lipids. Starch grains were also, in general, observed in the ray parenchyma cells of sapwood, disappeared with the death of the cells, and did not exist in heartwood.

Lipid generally showed droplet-like structure (Fig. 7 (a)). They showed reddish pink with Nile blue and red with Sudan IV in sapwood. The lipid droplets in sapwood, therefore, are thought to be neutral lipid judging from the color. Lipid droplets showed conspicuous changes at the boundary between sapwood and heartwood. That is, the color changed from reddish pink to blue in the sections stained with Nile blue (photograph is not shown) and the shape also changed from droplet-like to irregular shape (Fig. 7 (b)). These changes

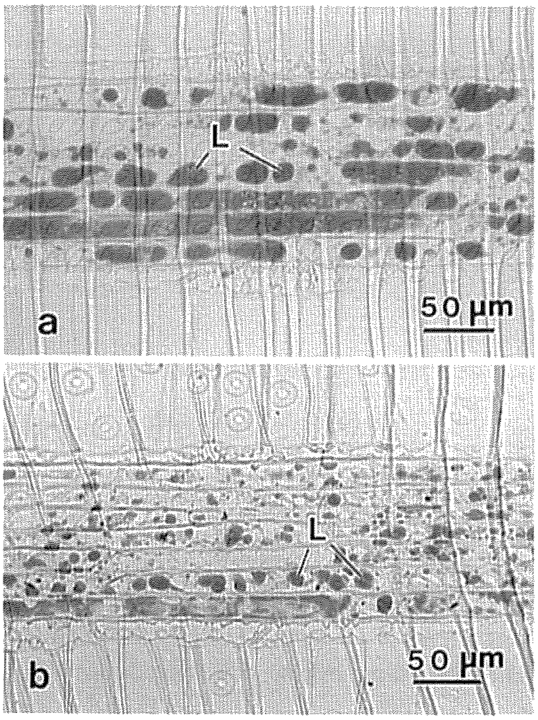


Fig. 7 Light micrographs of radial sections stained with Sudan IV showing lipid droplets (L) (Tree A, October). (a) Sapwood, (b) heartwood.

in lipids indicate the decrease in the volume of neutral lipids and the biochemical changes of neutral lipids to other materials. In the necrosis of ray parenchyma cells of *Pinus thunbergii* inoculated with pine wood nematodae, *Bursaphelenchys xylophilus*, it is reported that lipid droplets changed from reddish pink to blue[14]. The changes in lipid droplets in this experiment, therefore, are thought to have strong relationship to the death of ray parenchyma cells.

3.4 *Radial changes of moisture content*

Characteristics of water in the sapwood-heartwood boundary were investigated from the radial distribution patterns of moisture content. The results of Tree A (June and October) are shown in Fig. 8. In Fig. 8 the horizontal axis is based on the sample of June and two curves are adjusted at the boundary between

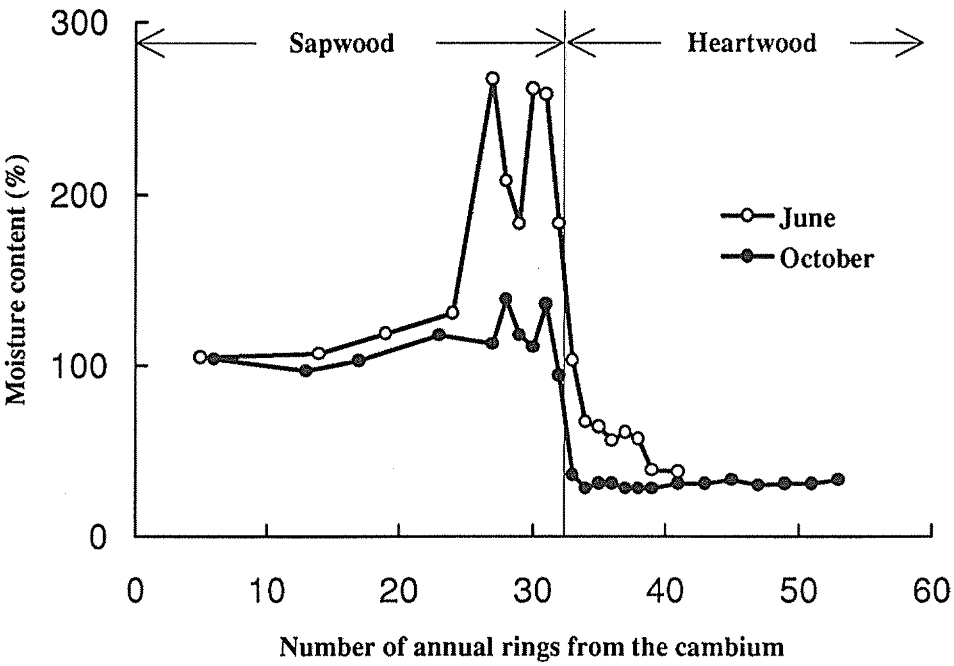


Fig. 8 Radial distribution patterns of moisture content (Tree A).

sapwood and heartwood.

The moisture content of sapwood, especially in the inner sapwood, showed higher value. It abruptly decreased at the sapwood and heartwood boundary and showed low values (about 40%) in heartwood. In Japanese cedar, the low moisture content in intermediate wood was the general pattern[15]. This is a very much different point from Japanese red pine which has no intermediate wood.

Comparing June and October, there was a conspicuous difference in moisture content in the inner sapwood. This difference in moisture content is considered to be the seasonal fluctuation of moisture content. If the drying of the tissue at the boundary between sapwood and heartwood is one of the necessary conditions for heartwood formation as Zycha[16] pointed out, the low moisture content in the inner sapwood in October is thought to contribute to heartwood formation.

3.5 *The characteristics of heartwood formation in Japanese red pine*

The characteristics of heartwood formation in Japanese red pine were discussed considering the relationship among the features mentioned above.

It was the noticeable characteristics that the abrupt changes in measured and observed points occurred in the transition zone from sapwood to heartwood. That is, the proportion of living ray parenchyma cells abruptly decreased from 100 to 0% in this zone together with the disappearance of starch grains and the changes of lipid droplets. Moisture content also decreased and heartwood phenols increased abruptly in very narrow region from sapwood to heartwood. In Japanese cedar it was reported that the changes of characteristics under discussion occurred in wide range of the intermediate wood which was generally composed of a few annual rings. Although an intermediate wood was not observed in Japanese red pine of present experiment, investigated changes in the transition zone of Japanese red pine, however, were similar qualitatively to those of Japanese cedar. We can conclude, therefore, that the zone of transition from sapwood to heartwood was very narrow in Japanese red pine but changes concerning heartwood formation were basically the same tendency as Japanese cedar which has a clear intermediate wood.

References

1) Nobuchi, T., Matsuno, H. and Harada, H. (1985) Radial distribution of heartwood phenols and the related cytological structure in the fresh wood of sugi (*Cryptomeria japonica* D. Don). Mokuzai Gakkaishi. **31** (9). 711-718

2) Nobuchi, T., Takahara, S. and Harada, H. (1979) Studies on the survival rate of ray parenchyma cells with aging process in coniferous secondary xylem. Bull. Kyoto Univ. Forests. No. **51**. 239-246

3) Nobuchi, T., Kamizono, Y. and Harada, H. (1976) Cytological changes of parenchyma cells associated with heartwood formation – On three softwood species, sugi, momi and akamatsu. Bull. Kyoto Univ. Forests. No. **48**. 178-186

4) Mibayashi, S., Satoh, K. and Yokota, T. (1985) Discoloration of wood by acid. Mem. Coll. Agric. Kyoto Univ. No. **127**. 49-59

5) Harris, J. M. (1954) Heartwood formation in *Pinus radiata* D. Don. New Phytol. **53**. 517-524

6) Hata, K. (1952) Studies on the pulp of "Akamatsu" (*Pinus densiflora* Sieb. st Zucc.) wood. XVI On the difficulty of sulphite digestion of the pine heartwood (5). Tech. Bull. Kagawa Agric. Coll. **4** (1). 8-13

7) Hayashi, H., Ohashi, H. and Yasue, Y. (1985) The relationship between the color of hinoki wood and hinokiresinol. Proc. 35th. Jap. Wood Res. Soc. 307

8) Hirai, S. (1951) Study on the process of heartwood growth in the Japanese larch stem. 59th. Meet. Jpn. Forests Soc. 231-234

9) Yamamoto, K., Fukazawa, K. and Ishida, S. (1978) Study on the cell wall development of ray parenchyma in genus *Pinus*. Report 2. The season of cell wall thickening and lignification in *Pinus strobus*. Res. Bull. Coll. Exp. Forest Hokkaido Univ. **35** (1). 99-108

10) Yamamoto, K., Fukazawa, K. and Ishida, S. (1979) Study on the cell wall development of ray parenchyma in genus *Pinus*. Report 3. Seasonal changes of cell wall thickening and lignification in *Pinus banksiana*. Ibid. **36** (2). 451-458

11) Rudloff, E. and Jorgensen, E. (1963) The biosynthesis of pinosylvin in the sapwood of *Pinus resinosa* Ait. Phytochem. **2**. 297-304

12) Ziegler, H. (1968) Biologische Aspekte der Kernholzbildung. Holz als Roh- und Werkstoff. **26** (2). 61-68

13) Nobuchi, T., Kuroda, K., Iwata, R. and Harada, H. (1982) Cytological study of the seasonal features of heartwood formation in sugi (*Cryptomeria japonica* D. Don). Mokuzai Gakkaishi. **28** (11). 669-676

14) Nobuchi, T., Tominaga, K., Futai, K. and Harada, H. (1984) Cytological study of pathological changes in Japanese black pine (*Pinus thunbergii*) seedlings after inoculation with pine-wood nematode (*Bursaphelenchus xylophilus*). Bull. Kyoto Univ. Forests. No. **56**. 224-233

15) Nobuchi, T. and Harada, H. (1983) Physiological features of the "white zone" of sugi (*Cryptomeria japonica* D. Don). Cytological structure and moisture content. Mokuzai Gakkaishi. **29** (12). 824-832

16) Zycha, H. (1948) Über die Kernbildung und verwandte Vorgänge im Holz der Rotbuche. Forstwissenschaft. Zentralb.. **67**. 80-109